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The α5β1 integrin selectively enhances epidermal growth factor signaling to the phosphatidylinositol-3-kinase/Akt pathway in intestinal epithelial cells

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Abstract

We have investigated EGF-driven signaling processes in rat intestinal epithelial cell lines that overexpress either the α5β1 integrin or the α2β1 integrin. Both cell types display efficient activation of Erk/MAP kinase, but only the α5β1 expressing cells display a strong activation of Akt. A complex is formed between activated EGFR and α5β1, but not with α2β1; this complex also contains ErbB3 and p85. Thus α5β1 can support efficient activation of both the Erk and the phosphatidylinositol-3-kinase/Akt branches of the EGFR signaling cascade, whereas α2β1 can support only the Erk branch.

Keywords: Integrin; Phosphatidylinositol-3-kinase; Akt/PKB; Epidermal growth factor receptor; Signaling

1. Introduction

During the last few years, abundant evidence has accumulated demonstrating that integrins and associated cytoskeletal components play a key role in permitting efficient signal transduction through the receptor tyrosine kinase (RTK)/Ras/MAP kinase pathway [1,2]. Integrins can modulate the ability of the pathway to respond to exogenous growth factors, and can also directly stimulate elements of this signaling cascade. Integrin regulation of the RTK/Ras/MAP kinase pathway is known to occur at several levels. Thus, there is good evidence that integrin engagement can enhance the efficiency of activation of RTKs [3,4] and, in some cases, can even trigger RTK activation in the absence of added growth factor [5]. In most cases, the integrin effect on RTK activation is accompanied by formation of a complex between the RTK and the integrin [4,6]. Integrin engagement can also influence efficiency of signal transmission between cytoplasmic components of the pathway by regulating the activation of Raf-1 [7,8] or of MEK [9].

The integrins are a complex family of heterodimeric molecules comprised of at least 18 distinct α subunits and eight β subunits [10]. While not all α/β heterodimers are permitted, there are about two dozen distinct integrins in vertebrates. This complexity

Abbreviations: PI-3-kinase, phosphatidylinositol-3-kinase; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; MAP kinase, mitogen activated protein kinase; p85, 85 kDa subunit of PI-3-kinase; RIE, rat intestinal epithelial cells

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engenders important questions concerning the possibility of specific roles for individual integrins in modulating signaling processes [11]. However, the effects of integrins on growth factor mediated activation of the MAP kinase cascade seem rather promiscuous, since a variety of integrin heterodimers are capable of enhancing this process [12]. Nonetheless, there is good evidence for unique roles in signaling and subsequent biological effects for some integrins. For example, the α6β4 integrin demonstrates some selective functions with respect to ErbB2 mediated signaling and carcinoma cell invasion [13–15]. Similarly, specific roles in regulating programmed cell death have been suggested for the α6β1 integrin in breast epithelial cells [16], and for the α5β1 integrin in several cell types [17,18].

We have recently re-examined the role of the α5β1 integrin in protection against programmed cell death [19]. Using a rat intestinal epithelial cell model, we demonstrated that over-expression of α5β1, but not of α2β1, provided dramatic protection against several pro-apoptotic stimuli. Further, the protective effects of α5β1 were mediated through a phosphatidylinositol-3-kinase (PI-3-kinase)/Akt dependent pathway. Overexpression of α5β1 substantially enhanced the ability of growth factors to signal to Akt [19], a kinase known to play an important role in opposing apoptosis [20].

In the current report we explore the mechanistic basis for enhanced signaling to Akt in rat intestinal epithelial cells that overexpress α5β1. We show that cells expressing either α5β1 or α2β1 are equally capable of activating the Erk/MAP kinase in response to growth factors such as EGF, but only cells expressing α5β1 are able to effectively activate Akt. The α5β1 effect primarily influences the maximal level of active Akt attained, rather than the concentration-response profile for the growth factor. Enhanced EGF-mediated activation of Akt in α5β1 cells is paralleled by increased autophosphorylation of EGF-receptor (EGFR, ErbB), and of ErbB3, and by increased association of the p85 subunit of PI-3-kinase with the ErbB family members. In addition, a multi-protein complex is formed between activated EGFR, ErbB3, p85 and α5β1, but not with α2β1. Thus α5β1 seems capable of selectively regulating the PI-3-kinase branch of the EGFR signaling pathway.

2. Materials and methods

2.1. Cell culture and lysis

Human α2- or α5-expressing RIE-1 cells (lines α2-P1 or α5-c10, respectively) were cultured as described in a previous study [19]. Cells were harvested by trypsinization, maintained in suspension for 45 min, and then replated on fibronectin- or collagen I-coated substrata for 1 h; EGF treatments at the indicated concentrations were performed for the last 5 min of the replating period. In some cases, cells were pretreated with LY294002, PP1 or PP2, or PD153035, which are selective inhibitors for PI-3-kinase, Src-kinase, and EGFR kinase respectively. Cell lysates for MAPK or PKB/Akt assays were prepared in the same manner as previously described [19]. Lysates for co-immunoprecipitations were prepared as described in a previous study [5].

2.2. Western blots

Western blots for MAPK or PKB/Akt activities in the lysates prepared from the cell lines were done with either anti-active MAPK (Promega, Madison, WI), anti-MAPK (Santa Cruz Biotech, Santa Cruz, CA), anti-pS473 PKB/Akt (Upstate Biotechnology, Lake Placid, NY), or sheep anti-rat PKB/Akt (New England Biolabs, Beverly, MA) polyclonal antibodies, as described previously [19]. Western blots for expression levels of EGFR, ErbB3, p85, Grb2, or β-tubulin in RIPA buffer lysates were performed with rabbit polyclonal anti-EGFR antibody (a gift from Dr. Shelton Earp, University of North Carolina, Chapel Hill, NC), monoclonal anti-ErbB3 antibody (Upstate Biotechnology), rabbit polyclonal anti-p85 antibody (Upstate Biotechnology), monoclonal anti-Grb2 antibody (Transduction Laboratory, Lexington, KY), or monoclonal anti-β-tubulin antibody (Boerhinger Mannheim, Germany), respectively.

It should be noted that, under the conditions used in most of the experiments reported here, ErbB family members appear in Western blots as two bands, both of which become tyrosine phosphorylated in response to EGF. This doublet is due to the initial trypsinization prior to replating the cells on collagen I or fibronectin. Thus, when unperturbed monolayers

are lysed and analyzed, the EGF receptor (and other ErbB proteins) run as a single band corresponding to the higher molecular mass component of the doublet seen in the trypsinized cells (data not shown).

2.3. Immunoprecipitations

For EGFR or ErbB3 immunoprecipitation, lysates with the same amount of protein in the same volume were incubated overnight at 4°C with sheep polyclonal anti-EGFR antibody, or monoclonal anti-ErbB3 antibody (Upstate Biotechnology), and protein-G-Sepharose beads (Calbiochem, San Diego, CA) were then added for a further 2 h. For co-immunoprecipitation of EGFR with integrin subunits, lysates with equal amounts of protein in equal volumes were immunoprecipitated with either anti-human α2 mAb (P1E6, Life Technologies, Rockville, MD) or anti-human α5 monoclonal antibody (MAB1996, Chemicon, Temecula, CA), by rolling at 4°C for 3 h. After addition of protein-G-Sepharose beads, immunoprecipitates were further incubated at 4°C for 1.5 h. After washing once with the cold lysis buffer and four times with ice-cold PBS, the immunoprecipitates were suspended with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and then boiled for 5 min. The immunoprecipitates were blotted with monoclonal anti-α5 antibody (Transduction Laboratory), rabbit polyclonal anti-α2 cytoplasmic tail antibody (a kind gift from Dr. G. Tarone, University of Turin, Turin, Italy), rabbit polyclonal anti-EGFR antibody, monoclonal anti-ErbB3 antibody, monoclonal anti-Grb2 antibody, rabbit polyclonal anti-p85 antibody, or monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology).

3. Results and discussion

In initial experiments we compared the ability of the α5β1 and α2β1 integrins to support growth factor mediated activation of MAP kinase and of Akt. Thus, RIE cells stably transfected with human α2 or α5 subunits were allowed to attach to substrata coated with the appropriate integrin ligands (collagen and fibronectin, respectively). The cells were then stimulated with various amounts of EGF, and the activation of MAP kinase and Akt determined. As seen in Fig. 1, both α5 cells and α2 cells displayed robust activation of MAP kinase in response to EGF; the maximal activities and the concentration versus response profiles were very similar. By contrast, only α5 cells displayed a robust activation of Akt. For both α5 cells and α2 cells, the maximal achievable activation of Akt was usually attained between 5 and 10 ng/ml of EGF; however, the maximal response attained in the α5 cells was approximately 4–5-fold greater than in the α2 cells. A similar differential response between α5 cells and α2 cells was also seen after longer periods of treatment with EGF; thus the result cannot be attributed to a delayed response (data not shown).

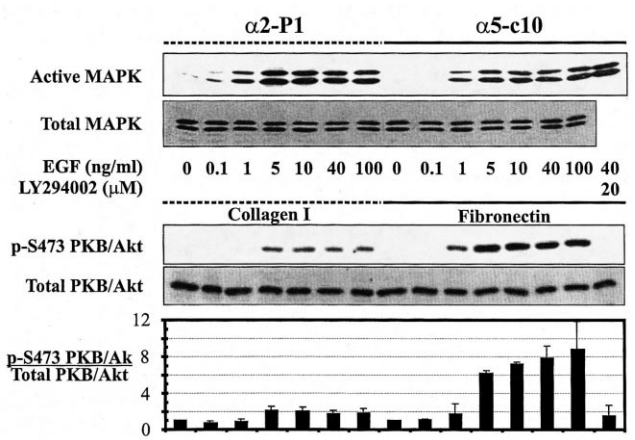
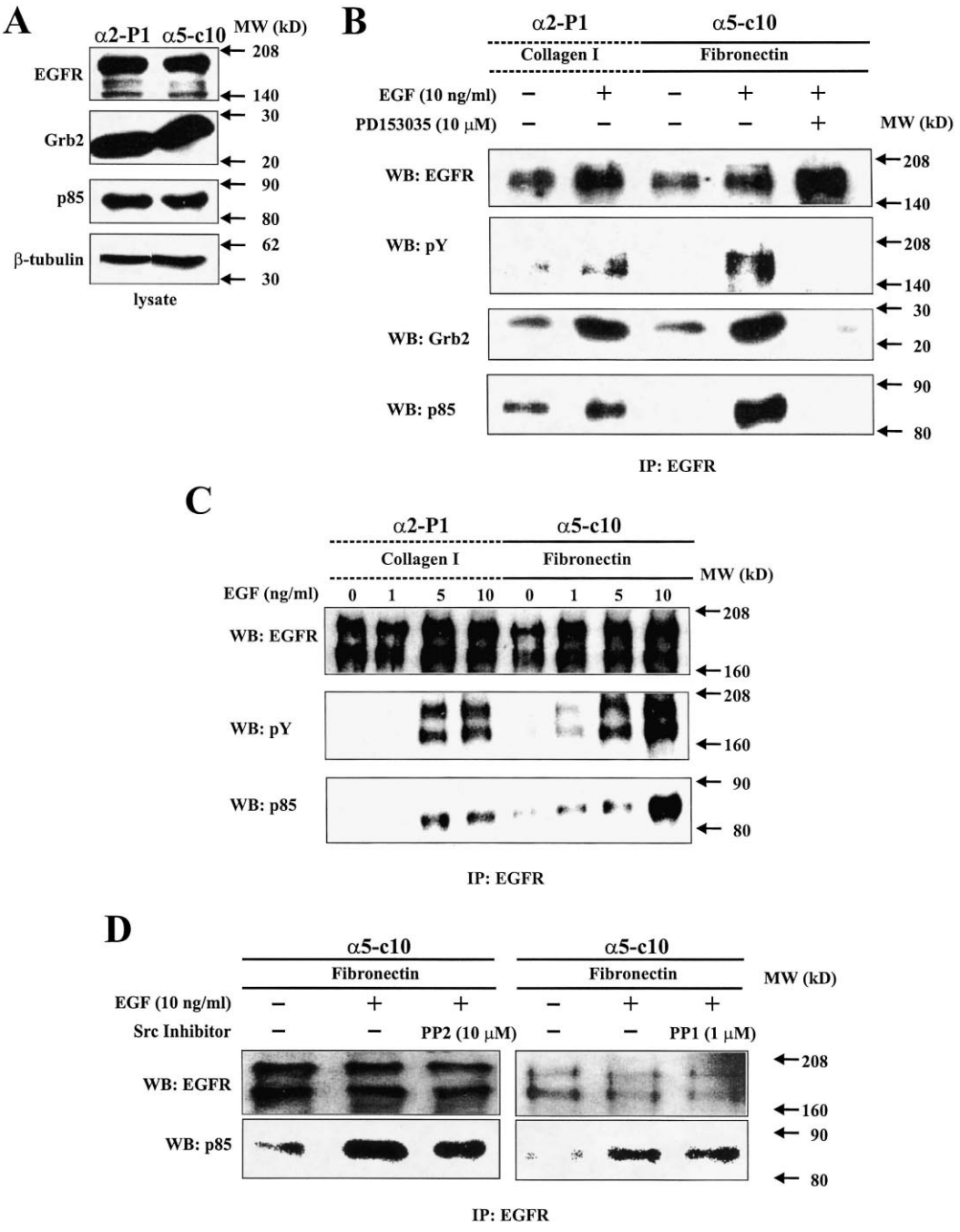


Fig. 1. EGF dose response for PKB/Akt and MAPK activation. RIE α2-P1 or α5-c10 cells were replated on collagen I (20 μg/ml) or fibronectin (20 μg/ml) for 1 h, respectively, and were treated for the last 5 min with EGF at various concentrations prior to harvest. In some cases, cells were pretreated with LY294002 at 20 μM. The lysates were analyzed in Western blots using anti-active MAPK, anti-MAPK, anti-phospho-PKB/Akt (p-Ser473), or anti-PKB/Akt antibody. The band intensities of PKB/Akt blots were quantitated by using a chemifluorescence-based method (Amersham Pharmacia Biotech), as explained in a previous study [19]. The graph shows the ratio of the band intensity of Ser473-phosphorylated PKB/Akt over that of total PKB/Akt at the corresponding condition. The data shown are representative of five independent experiments.

Receptor tyrosine kinases signal to multiple downstream effectors via complex, branched pathways [21,22]. The initiation of any one branch of the overall response often entails the binding of an SH2 domain adaptor protein to the activated, autophosphorylated RTK [23]. For the MAP kinase path, Grb2 serves as a key adaptor, while for the PI-3-ki-



nase/Akt branch the p85 subunit of PI-3-kinase is essential. Thus, to further evaluate the ability of α5β1 to selectively enhance growth factor signaling to Akt, we examined the binding of Grb2 and of p85 to EGF receptor in both α5 cells and α2 cells.

Initially we examined the total cellular pools of the various signaling components. Thus, as seen in Fig. 2A, there were comparable amounts of EGFR, Grb2 and p85 expressed in α5 cells and α2 cells. As seen in Fig. 2B, however, there was enhanced autophosphorylation of EGFR in α5 cells as compared to α2 cells, as revealed by anti-phosphotyrosine Western blots. This was accompanied by increased association of p85 with the EGFR complex in α5 cells, as demon-

Fig. 2. Differential formation of signaling complexes downstream of the EGFR in cells expressing different α integrin subunits. α2-P1 or α5-c10 cells were replated and treated with EGF at the indicated concentrations, in the same manner as described in Fig. 1. In some cases, cells were pretreated with PD153035 at 10 μM. The data shown are representative of several experiments. (A) Expression of signaling components. Total cell lysates prepared with RIPA buffer were Western blotted for EGFR, Grb2, p85, or tubulin. (B) Differential autophosphorylation of EGFR and signaling complex formation in α5-c10 cells. The lysates were immunoprecipitated with anti-EGFR antibody and the immunoprecipitates were blotted for EGFR, phosphotyrosine, Grb2, or the p85 regulatory domain of PI-3-K. After the proteins were transferred from a gel to a PVDF membrane, the membrane was cut into two pieces. The upper part with higher molecular mass proteins was used for blotting for EGFR or phosphotyrosine, whereas the lower part was used for blotting for p85 or Grb2. (C) EGF dose-dependent EGFR autophosphorylation and p85 association with EGFR. The lysates and EGFR immunoprecipitates were prepared as described above. The upper parts (>120 kDa) of gels (6% SDS–PAGE) were blotted for EGFR or phosphotyrosine, whereas the lower parts (<120 kDa) were blotted for p85. (D) Src does not affect p85 association with EGFR. Cells were treated with EGF and with the Src-selective inhibitors PP1 or PP2, or not. Immunoprecipitates of EGFR were analyzed for total EGFR and for p85. The positions of molecular mass markers are shown with arrows. The experiments shown in this figure were repeated at least twice.
←

strated by co-immunoprecipitation. By contrast, the binding of Grb2 to the EGFR was similar in both cell types. The selective inhibitor PD153035 dramatically reduced EGFR autophosphorylation, and thus p85 and Grb2 association, as expected. The differences in EGFR autophosphorylation and p85 recruitment are more clearly revealed when EGF concentration–response profiles are examined. As seen in Fig. 2C, in parallel to the Akt response of Fig. 1, maximal attainable P85 association with EGFR was reached at approximately 10 ng/ml of EGF; however, the maximal amount of p85 recruitment was substantially greater in the α5 cells as compared to the α2 cells. Thus in the α5 cells more phosphorylated EGFR and more p85 was found in the EGFR immunocomplex. It has been suggested that binding of p85 to EGFR can be increased subsequent to Src-mediated tyrosine phosphorylation of the EGFR cytoplasmic domain [24]. However, treatment of α5 cells with the selective Src kinase inhibitors PP1 or PP2 did not affect association of p85 with EGFR (Fig. 2D), suggesting that Src is not involved in this system.

While the binding motifs for Grb2 on the EGFR cytoplasmic domain have been well defined, binding sites for p85 are not obvious. Many of the complex signaling properties of EGFR (ErbB) are thought to occur via its heterodimerization with the other three members of its family (ErbB2, 3, 4) [21,25]. It has been suggested that PI-3-kinase activation in response to EGF treatment takes place primarily via heterodimerization of EGFR with ErbB3, which has several p85 consensus binding sites, although it is not an active kinase itself [26,27]. Thus, EGFR has Grb2 docking sites at positions 1068 and 1086 but no p85 binding sites, while ErbB3 has p85 sites at positions 1035, 1178, 1203, 1241, 1257, and 1270, but no Grb2 sites [28]. Another possibility is the adaptor protein c-Cbl, which preferentially associates with EGFR [29], and which provides a docking site for p85 [30].

To explore these issues, we examined the possibility of enhanced heterodimerization or activation of ErbB3 in response to EGF. As seen in Fig. 3A, Western blotting of cell lysates revealed that α2 cells actually contained more ErbB3 than did the α5 cells. In immunoprecipitates of total EGFR (Fig. 3A) the amount of ErbB3 increased upon EGF treatment, indicating preferential association of the activated EGFR and ErbB3 receptors, as expected [21]. Further, the amount of ErbB3 in the EGFR immunocomplex was higher in the α5 cells than in α2 cells, despite the fact that more ErbB3 is found in the α2 cells. We also examined the tyrosine phosphorylation of ErbB3 and its association with p85. As seen in Fig. 3B, there was significantly more tyrosine phosphorylation of ErbB3 and more associated p85 in the ErbB3 immunoprecipitates from the α5 lysates as compared to the α2 lysates. Thus there seems to be enhanced formation of an EGFR/ErbB3/p85 complex in the α5 cells in response to EGF. We also tested for the presence of c-Cbl in the EGFR complex. Treatment with EGF triggered increases in c-Cbl association with the EGFR complex, but the increase was similar in the α5 cells and α2 cells (data not shown). Thus, enhanced binding of c-Cbl per se does not explain the positive influence of α5β1 on PI-3-kinase-dependent Akt activation.

We next explored the possibility of a selective as-

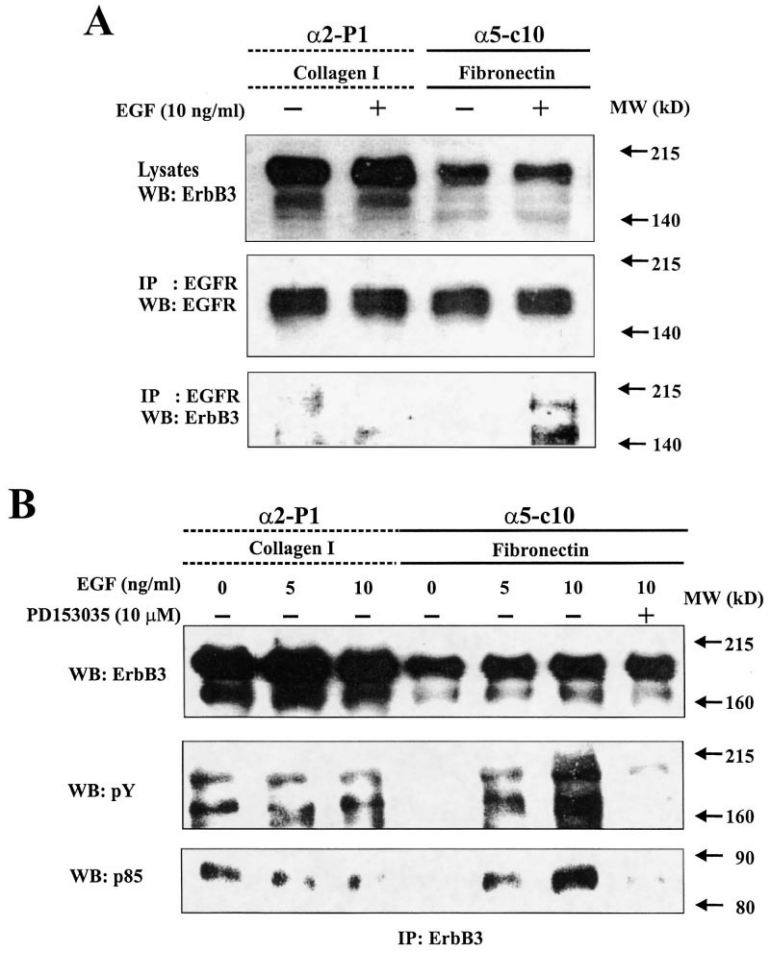


Fig. 3. Heterodimerization of EGFR and ErbB3. (A) Co-immunoprecipitation of ErbB3 with EGFR. α2-P1 or α5-c10 cells were re-plated on collagen or fibronectin respectively and treated with EGF as indicated. Cell lysates were Western blotted for total ErbB3 content. EGFR immunoprecipitates were Western blotted for EGFR and for ErbB3. (B) Co-immunoprecipitation of p85 with ErbB3. The experimental conditions were similar to those of A except that anti-ErbB3 was used to form an immune complex, which was then Western blotted for phosphotyrosine and for p85. The positions of molecular mass markers are shown with arrows. The data shown are representative of at least three different experiments.

sociation between ErbB family members and the α5β1 integrin. As seen in Fig. 4A, EGFR was co-immunoprecipitated by anti-α5 antibody in α5 cells treated with EGF, but not by anti-α2 antibody in α2 cells similarly treated. Exposure of cells to the EGFR selective kinase inhibitor PD153035 blocked the ability of EGFR to co-immunoprecipitate with the α5β1 integrin. Reciprocal experiments precipitating with anti-EGFR and then Western blotting for integrin subunits confirmed the EGFR/α5β1 linkage (Fig. 4B). Thus, a fraction of activated EGFR was able to selectively associate with the α5β1 integrin while virtually no association with α2β1 was observed. Similarly, as seen in Fig. 4C, the ErbB3 receptor preferentially associated with α5β1 in the α5 cells treated with EGF, despite the fact that more ErbB3 is found in the α2 cells.

In summary, our results indicate that a fraction of activated EGFR forms a complex with α5β1 integrin, and that this complex also includes tyrosine phosphorylated ErbB3. The ability of phosphorylated ErbB3 to bind the SH2 domain of p85 results in enhanced recruitment of p85 to the EGFR complex. The increased recruitment of p85 correlates well with the higher levels of Akt activation seen in the α5 positive cells, and may thus substantially contribute to the anti-apoptotic effects of α5β1 overexpression that we have previously observed [19]. By contrast,

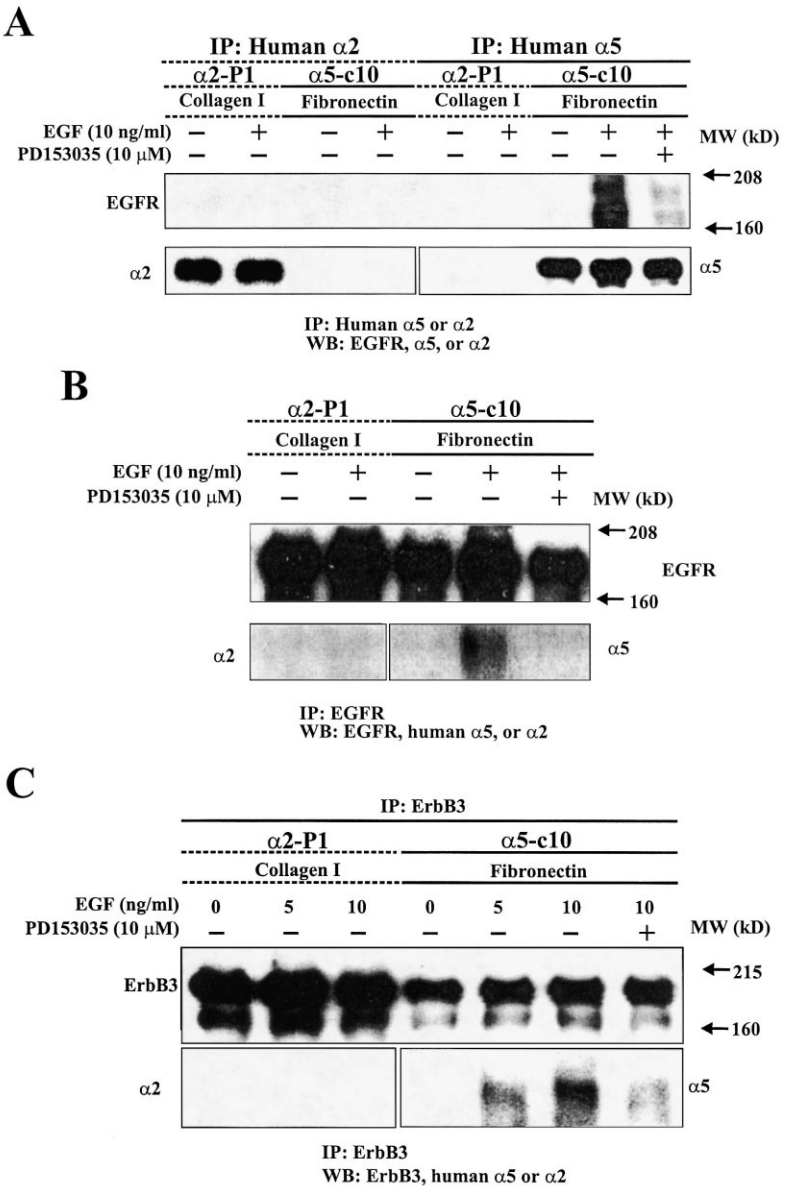


Fig. 4. Co-immunoprecipitation of activated EGFR with α5, but not with α2, integrin subunit. The lysates and co-immunoprecipitations with anti-human α2, α5, or EGFR were prepared as described in Section 2. In some cases cells were pretreated with PD153035 at 10 μM prior to being replated on substrates. (A) Co-immunoprecipitation of activated EGFR with anti-human α5 antibody. The α5 and α2 immunoprecipitates were blotted for anti-EGFR, and then stripped prior to blotting with either human α2 or α5 subunit antibody. (B) Reciprocal co-immunoprecipitation of human α5 integrin subunit with anti-EGFR antibody. The immunoprecipitates using anti-EGFR were blotted for integrin subunits and then stripped prior to re-blotting with anti-EGFR antibody. (C) Co-immunoprecipitation of α5 subunit with ErbB3. Lysates from EGF treated cells were immunoprecipitated with anti-ErbB3. The immune complexes were Western blotted with anti-ErbB3, anti-α5 or anti-α2 antibodies. The positions of molecular mass markers are shown with arrows. The experiments shown in this figure were repeated at least twice.

the recruitment of Grb2 and activation of the Erk/MAP kinase pathway is similar in both α5 cells and α2 cells. It seems that the level of tyrosine phosphorylation of EGFR attained in either cell type is sufficient to promote full activation of the Erk pathway. However, full activation of the PI-3-kinase/Akt pathway may require the enhanced recruitment and tyrosine phosphorylation of ErbB3 seen in the α5β1 cells.

At this point it is not clear if α5β1 primarily interacts with EGFR, with ErbB3, or with both proteins. Similar selective association of a particular integrin with growth factor receptors has been demonstrated previously for the case of αvβ3 associating with PDGFR [6] and VEGFR [4]. For the EGFR, previous work indicated that EGFR associates with β1 integrins [5], but the α subtype specificity had not been defined. It should be noted that our studies on the α5β1 integrin do not preclude the possibility that other integrin α/β heterodimers may also be efficient in supporting PI-3-kinase/Akt signaling. However, α2β1 clearly does not have this ability to the same degree as α5β1; thus there seem to be distinct sub-sets of β1 integrins in terms of RTK activation and effects on PI-3-kinase signaling. It should be noted that other investigators have suggested that the EGF-R can complex with α2β1 to some degree; however, in this instance no direct comparison with α5β1 was made [31]. Another study has linked α5β1 protection against apoptosis to a pathway that involves focal adhesion kinase (FAK), Shc, PI-3-kinase, Akt and Bcl-2 [32]. This study may be complementary to the present work because of the possibility of interplay between FAK signaling and RTK signaling.

The precise mechanism of the RTK/integrin association is still unclear. It is not known whether the association is direct, or whether other proteins assist in the formation of a complex between the integrin and the RTK. It has been suggested recently [33] that the cooperation between integrins and growth factor receptors may be based on formation of a complex between the adhesion-activated intracellular tyrosine kinase FAK and the cytoplasmic domains of the receptor tyrosine kinases. However, another recent study [34] has indicated that the association between αvβ3 and PDGF receptor or VEGFR2 is via the external domain of β3, rather than the cytoplasmic domain. Thus, the precise mechanism(s) of integrin/RTK interaction remain somewhat controversial. In the present case we have shown that the α5β1 integrin is much more effective than the α2β1 integrin in supporting formation of an EGFR/ErbB3/P85 complex in intestinal epithelial cells. These findings provide a basis for understanding the selective effects of α5β1 in supporting enhanced activation of the PI-3-kinase/Akt signaling pathway and its role in inhibiting apoptosis.

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